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Dear

I enclose a brief outline of the soluble tumor necrosis factor receptor project that we discussed last Monday. Included also are budgets for the first two specific aims. These budgets describe the funding necessary to complete these initial aspects of the work as we discussed it here at CSU. However I failed to take into account, at that time, the need to include 15% indirect costs per university regulations; hence, the increase from an estimated to nearly

I have attempted to define the timeframes in which the various stages of the work can be completed. As indicated, input from the clinicians will be essential to firmly determine the time needed for the clinical studies.

I think that the project has significant promise. It can be completed with modest outside funding, since we have the luxury of working through the university. Moreover, the proposed timeframe is relatively short. If all were to proceed as planned, an application to conduct Phase I human trials could be submitted as early as . Additionally, the ability to develop products for canine oncology along the way is an added bonus. Please call me if you would like to discuss the enclosed materials or any other aspects of the project.

I enjoyed meeting you, and I am confident that we could work together to accomplish these objectives. Thanks for lunch. I hope to hear from you soon.

Best regards,

Mark D. Howell, Ph.D.
Assistant Professor of Microbiology

Enclosures

IMMUNOSORBENT DEPLETION OF sTNFR FOR CANCER THERAPY
Project Summary

Aim 1 To demonstrate that sTNFR enhances tumor survival

Described in accompanying grant proposal to the College Research Council

Aim 2 To produce monoclonal antibodies to canine sTNFR

Task 1--Determine the level of cross-reactivity between human and canine sTNFR. The ELISA assay developed in Aim 1 above uses a polyclonal goat anti-human sTNFR antibody preparation. We will use this ELISA to screen sera from tumor-bearing dogs for the presence of molecules that are reactive with the anti-human sTNFR antibody. Detection of such molecules is likely, due to the highly conserved amino acid sequence of sTNFRs from different species. For example, human, mouse and rat sTNFR molecules exhibit 66% amino acid identity and 92% amino acid homology.

Task 2--To affinity purify canine sTNFR. Demonstration that the anti-human sTNFR antibody binds to canine sTNFR will allow the use of that antibody for the purification of canine sTNFR. Anti-human sTNFR will be immobilized on a solid support to create an affinity column. This column will be used to purify canine sTNFR from sera of tumor-bearing dogs shown by ELISA to contain immunologically cross-reactive molecules. Sera for these studies will be obtained from Greg Ogilvie at the CSU Veterinary Teaching Hospital.

Task 3--To verify that the immunoreactive molecules isolated from canine sera are sTNFR. The affinity-purified proteins will be characterized to insure that bona fide canine sTNFR has been isolated. Amino-terminal protein sequence of the affinity-purified molecules will be determined at the CSU Macromolecular Resources Center. Similarity between this sequence with that of the known sTNFR amino acid sequences will allow us to positively identify the isolated molecule as sTNFR. Immunoblot analysis also will be conducted to estimate the molecular weight of the isolated molecule.

Task 4--To produce monoclonal antibodies to canine sTNFR. Eight to ten week-old female Balb/c mice will be immunized with sTNFR emulsified in complete Freund's adjuvant. One month later, a booster in incomplete Freund's adjuvant will be administered. One month after that, an intravenous booster in saline will be administered and three days later the mice will be sacrificed and their spleens will be harvested. Splenocytes will be fused to the Balb/c myeloma, SP2/0, us~~ing~~ standard methods. Hybridomas will be selected and will be screened for immunoglobulin secretion by direct ELISA. Immunoglobulin-secreting hybridomas will be screened further for the production of anti-sTNFR antibodies by direct ELISA.

Aim 3 To develop sTNFR ELISA assays for early detection of canine cancer

Task 1--To incorporate monoclonal anti-canine sTNFR antibodies into an ELISA format for the detection of sTNFR. Two high-affinity monoclonal antibodies, reactive with non-overlapping determinants of sTNFR, will be identified. These antibodies will be used to develop an ELISA, similar in format to that described in Aim 1, which will be optimized for sensitivity and specificity. Calibration of the assay will be accomplished using the sTNFR purified from the sera of tumor-bearing dogs as a standard.

Task 2--To use the ELISA to correlate serum sTNFR levels with tumor development. The levels of sTNFR in sera from several dogs, with a variety of different tumor types, will be determined using the ELISA described above. Sera from non-tumor-bearing animals also will be analyzed. sTNFR values will be analyzed relative to tumor type, size and duration of tumor burden, genetic background (where available), gender, and additional clinically-relevant parameters. The significance of the observed correlations (p values) will be determined using Fisher's exact test, and the significance of variations in sTNFR levels will be assessed using ANOVA. It will be possible to obtain the large numbers of samples needed to statistically validate the results through the involvement of canine oncologists at the CSU Veterinary Teaching Hospital. Positive correlations between tumor development and increased sTNFR levels will provide the basis for the development of a commercial canine cancer screening assay and will justify the development of immunosorbent methods for the therapeutic removal of canine sTNFR.

Aim 4 To develop and test immunosorbent methods for the selective removal of sTNFR from sera or ultrafiltrates obtained from tumor-bearing dogs.

High-affinity monoclonal antibodies will be chemically coupled to a solid support. The matrix of choice is Actigel ALD Superflow (Sterogene Bioseparations, Inc., Arcadia, CA). This matrix offers simple, efficient and well-defined coupling chemistry, very high binding capacity for antibodies (up to 50 mg/ml), very high stability profiles (leaching at less than 0.1 ppm), and high flow rates (up to 3 L/hr). Actigel is currently in use in pharmaceutical processes, and Drug Master Files are available for both domestic and international applications. Several different antibodies will be coupled to Actigel and the resulting matrices will be tested for the ability to efficiently deplete sera and ultrafiltrates (see Aim 5) of sTNFR. Depletion experiments will be conducted on untreated sera and also on sera spiked with TNF to examine the influence of TNF-sTNFR complex formation on the depletion efficiencies. Antibody columns which effectively remove sTNFR from sera and which are not appreciably affected by TNF binding to sTNFR will be further evaluated in *in vivo* studies.

Aim 5 To evaluate the therapeutic benefit of ultrafiltration in canine oncology.

The most expeditious path to using immunosorbent matrices will be to incorporated them into the existing ultrapheresis technology to deplete the ultrafiltrate of sTNFR, thus allowing reinfusion of the ultrafiltrate and obviating the need for replacement fluid. As a prelude to such studies, the efficacy of the existing ultrapheresis methodology needs to be evaluated. Clinical trials will be conducted on tumor-bearing dogs treated at the VTH. The canine oncology group treats a large number of animals and routinely evaluates experimental therapies. has expressed an interest in beginning such studies, provided that the appropriate hardware (dialysis equipment and ultrafilters) can be provided. This trial will determine whether or not ultrapheresis enhances the survival of dogs with tumors, will provide ultrafiltrates from treated animals for analysis of sTNFR levels, and will establish clinical conditions necessary for future evaluation of immunosorbent methods.

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*The sooner the ultrapheresis hardware is in place, the sooner these studies can be completed. I hesitate to firmly commit to a timeline without consulting with the clinicians who will conduct the work. However, I would guess that a one year

study will generate sufficient data to justify further development efforts. If this study begins in the summer of , its completion will coincide with the development of the immunosorbent matrix which is estimated to be completed by

Aim 6 To evaluate the therapeutic benefit of the combined immunosorbent/ultrafiltration method in canine oncology.

An additional loop will be created in the existing ultrapheresis hardware into which the immunosorbent matrix will be incorporated. This will allow the ultrafiltrate to be depleted of sTNFR and returned to the animal. Clinical benefit derived from this treatment will prove the value of a biotherapeutic approach that removes specific inhibitors of the anti-tumor immune response, thus providing the basis for the development and testing of immunosorbent methods for removal of other inhibitors of the immune system. More importantly, positive data from this canine trial will be used to petition the FDA for approval to begin toxicity trials in human cancer patients. Conduct of such trials will require the development of immunosorbent matrices for human sTNFR, producing those matrices under Good Manufacturing Practice (GMP) guidelines, and the identification of clinical sites at which the studies will be conducted. The details of such an endeavor are beyond the scope of this document, however, I think the path leading to human studies is straightforward and will parallel the canine studies described above.

*Input from the clinicians will be required as in Aim 5.

BUDGET AIM 1

1. SALARY EXPENSE

2. OPERATING EXPENSES

Serologic reagents
Molecular biologics
Cell culture media and supplements
Plasticware
Animal purchase

3. TRAVEL EXPENSE

Travel funds for P.I.
to attend one national meeting

4. OTHER DIRECT EXPENSE

Animal care

Total Direct Costs	$
Indirect Costs (15%)	
TOTAL BUDGET	$

Budget justification: Half salary is requested for my research associate, , who will conduct the proposed research. ias 15 years of laboratory experience and is proficient in antibody production, purification and modification, ELISA and immunoblot assay development, molecular cloning and expression studies, and animal experimentation.

Funds are requested for serologic reagents including goat anti-sTNFR, alkaline phosphatase-conjugated rabbit anti-goat IgG, alkaline phosphatase-conjugated streptavidin, reagents for antibody biotinylation, and miscellaneous substrates and cofactors. Molecular biological reagents required include oligonucleotide primers, reagents for PCR amplification, the cloning vector pcDNA3, and various restriction and modifying enzymes for the production and selection of recombinant DNAs. Cell culture media and supplements, drugs, and disposable cultureware are requested for maintenance and selection of L929 cells and transfectants.

Funds are requested also for the purchase and care of C3H/HeN mice. Costs were calculated as follows:

BUDGET AIM 2

1. SALARY EXPENSE

2. OPERATING EXPENSES

Serologic reagents
Cell culture media and supplements
Plasticware
Animal purchase

3. TRAVEL EXPENSE

Travel funds for P.I.
to attend one national meeting

4. OTHER DIRECT EXPENSE

Animal care
Protein sequencing

Total Direct Costs	$
Indirect Costs (15%)	
TOTAL BUDGET	$;

Budget justification: Half salary is requested for my research associate, who will conduct the proposed research. has 8 years of laboratory experience and is proficient in monoclonal antibody production, characterization and purification.

Funds are requested for serologic reagents including fluorescein and alkaline phosphatase-conjugated anti-mouse immunoglobulins and miscellaneous substrates and cofactors. Cell culture media and supplements, drugs, and disposable cultureware are requested for production, selection and maintenance of hybridomas.

Funds are requested also for the purchase and care of Balb/c mice. Costs were calculated as follows:

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